EXHIBIT 2

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Epitope Mapping of CCR5 Reveals Multiple Conformational States and Distinct but Overlapping Structures Involved in Chemokine and Coreceptor Function*

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The chemokine receptor CCR5 is the major coreceptor for R5 human immunodeficiency virus type-1 strains. We mapped the epitope specificities of 18 CCR5 monoclonal antibodies (mAbs) to identify domains of CCR5 required for chemokine binding, gp120 binding, and for inducing conformational changes in Env that lead to membrane fusion. We identified mAbs that bound to N-terminal epitopes, extracellular loop 2 (ECL2) epitopes, and multidomain (MD) epitopes composed of more than one single extracellular domain. N-terminal mAbs recognized specific residues that span the first 13 amino acids of CCR5, while nearly all ECL2 mAbs recognized residues Tyr-184 to Phe-189. In addition, all MD epitopes involved ECL2, including at least residues Lys-171 and Glu-172. We found that ECL2-specific mAbs were more efficient than NH_2- or MD-antibodies in blocking RANTES or MIP-1β binding. By contrast, N-terminal mAbs blocked gp120-CCR5 binding more effectively than ECL2 mAbs. Surprisingly, ECL2 mAbs were more potent inhibitors of viral infection than N-terminal mAbs. Thus, the ability to block virus infection did not correlate with the ability to block gp120 binding. Together, these results imply that chemokines and Env bind to distinct but overlapping sites in CCR5, and suggest that the N-terminal domain of CCR5 is more important for gp120 binding while the extracellular loops are more important for inducing conformational changes in Env that lead to membrane fusion and virus infection. Measurements of individual antibody affinities coupled with kinetic analysis of equilibrium binding states also suggested that there are multiple conformational states of CCR5. A previously described mAb, 2D7, was unique in its ability to effectively block both chemokine and Env binding as well as coreceptor activity. 2D7 bound to a unique antigenic determinant in the first half of ECL2 and recognized a far greater proportion of cell surface CCR5 molecules than the other mAbs examined. Thus, the epitope recognized by 2D7 may represent a particularly attractive target for CCR5 antagonists.

Cellular entry by HIV-1[1] requires the presence of both CD4 and certain members of the chemokine receptor family (1–5). While numerous molecules can serve as coreceptors for one or more virus strains, the chemokine receptors CCR5 and CXCR4 are clearly the major coreceptors for R5 and X4 virus strains, respectively (6, 7). R5 virus strains are largely responsible for virus transmission, and individuals who lack CCR5 due to a natural knock-out mutation in the CCR5 gene (*ccr5 Δ32* allele) are highly resistant to HIV-1 infection (8–10). The importance of CCR5 for viral entry and replication is further underscored by the observation that individuals heterozygous for the *ccr5 Δ32* allele have a 2–4-year delayed progression to AIDS (8, 9, 11, 12), most likely due to reduced expression levels of CCR5 (13, 14). On the other hand, X4 viruses tend to emerge years after infection, and the switch from R5 to X4 viruses correlates with progression to AIDS (15–19).

The HIV-1 envelope (Env) glycoprotein is proteolytically processed from a gp160 precursor to form a mature noncovalent multimeric complex of gp120/41 subunits. Binding of gp120 to CD4 triggers conformational changes in Env that enable it to interact with the appropriate coreceptor (20–23). A highly conserved site in gp120 has been implicated in CCR5 binding (24), while the V3 loop as well as the V1/2 region have been shown to play major roles in the choice of coreceptor used by a given virus strain (25–29). Coreceptor binding is thought to lead to additional conformational changes in Env that result in exposure of the hydrophobic fusion peptide in gp41, which mediates mixing of the viral and cellular membranes (1–5). Thus, coreceptors support both Env binding and conformational change induction.

Chemokine receptors are members of the seven-transmem-

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[1] The abbreviations used are: HIV, human immunodeficiency virus; mAb, monoclonal antibody; FACS, fluorescence-activated cell sorting; MCF, mean channel fluorescence; PBS, phosphate-buffered saline; BSA, bovine serum albumin; MD, multidomain; HA, hemagglutinin; RANTES, regulated on activation, normal T-cell expressed and secreted.

brane G-protein-coupled receptor family. Seven-transmembrane G-protein-coupled receptors have complex membrane topologies consisting of an N-terminal domain, three extracellular and intracellular loops, and a cytoplasmic C-terminal tail. Studies with mutant and chimeric coreceptors have indicated that Env interactions with CCR5 are complex, requiring cooperativity between multiple extracellular domains and with multiple residues on the chemokine receptor (1, 4, 30). Further, there are differences in how virus strains interact with CCR5, with R5X4 strains being particularly sensitive to alterations in the N-terminal domain of CCR5 (31–34). However, whether CCR5 domains involved in Env binding and in conformational change induction are distinct, overlapping, or identical is not yet clear.

In this paper, we generated a large panel of anti-CCR5 monoclonal antibodies (mAbs) to probe the structural determinants of CCR5 chemokine receptor and coreceptor function. We mapped the epitopes of these antibodies to the extracellular domains of CCR5 using chimeric and mutant receptors, and correlated this information with the ability of these mAbs to block chemokine and HIV-1 Env binding as well as coreceptor function. We found that CCR5 has several immunodominant epitopes on its extracellular face and provide evidence for distinct but overlapping sites on CCR5 for chemokine and Env binding. mAbs to the second extracellular loop (ECL2) of CCR5 were more effective at blocking chemokine than Env binding, while mAbs to the N-terminal domain blocked gp120 binding but had little effect on CCR5-chemokine interactions. Despite this, antibodies to ECL2 more effectively neutralized virus than did antibodies to the N terminus of CCR5. These results suggest that the N-terminal domain of CCR5 is more important for Env binding, while the extracellular loops are more important for inducing the conformational changes in Env that lead to membrane fusion. Measurement of the relative affinities of these mAbs for CCR5 combined with analysis of equilibrium binding kinetics suggested that there are multiple conformational states of CCR5 on the cell surface. Finally, the epitope maps of these mAbs were used to provide constraints on a theoretical model of CCR5 structure.

MATERIALS AND METHODS

Plasmids—All constructs unless otherwise specified were made in pcDNA3. Plasmids expressing the CCR5/CCR2 chimeras have been described previously (31). The additional chimeras 55(2/5)5 and 55(5/2)5 were constructed by replacing a *Cla*I-*Eco*RI cassette in wild-type CCR5 with a fragment generated by polymerase chain reaction as described earlier for N-terminal domain chimeras (31). The junction between CCR5 and CCR2 sequences is at the conserved cysteine (Cys-178) that is presumably involved in a disulfide bond with the first extracellular loop. The mutant cassettes were transferred as needed into other backgrounds, such as CCR2b or the (5/2)222 chimera. The generation of the alanine scan mutants of CCR5 have also been described previously (32). Alanine scan mutants not previously reported were generated by site-directed mutagenesis using the Quick Change site-directed mutagenesis kit (Stratagene). All final constructs were verified by sequencing before their use in transfection experiments.

Antibodies—mAbs 45501, 45502, 45517, 45519, 45523, 45533, 45529, 45531, and 45549 (R&D Systems, Minneapolis, MN) were generated by immunizing BALB/c mice using syngeneic mouse myeloma (NSO) cells stably transfected to express the full-length human CCR5 sequence. An immunization protocol for soluble protein (35) was adapted for use with whole cells as the immunogen. Between 0.5 and 2 million viable transfected cells were injected on days 0, 3, 7, 10, and 14. The priming immunization was done by mixing the cell suspension in PBS with an equal volume (50 μl) of emulsified MPL/TDM adjuvant (Ribi); subsequent boosts used cells in 50 μl of PBS alone. Following immunization, popliteal lymph node cells were used for polyethylene glycol-mediated fusion. The hybridoma supernatants were then screened by cell-surface enzyme-linked immunosorbent assay (10^5 cells/well) for the presence of antibodies that bound to the immunizing cells. The positive subset was further tested on a panel of untransfected and irrelevantly transfected cells for specificity and finally confirmed by FACS analysis on the unfixed immunizing cells. The panel of candidates was subcloned two or three times each before extensive testing was performed on purified antibody derived from ascites. mAbs CTC2, CTC5, CTC8, CTC9, and CTC12 (PDL, Inc., Fremont, CA) were generated from BALB/c mice with CHO-CCR5 transfectants as immnogens using intraperitoneal injections every 2 weeks for 2 months; the first injection was performed with complete Freund's adjuvant. mAbs mCR35.4 and mCR40.3 (PDL, Inc.) were generated from AKR mice with NSO-CCR5 transfectants using footpad immunizations every 3 days for 21 days; the first injection was performed with Ribi adjuvant. Fusion was performed as described using spleen cells and lymph nodes from BALB/c and AKR mice, respectively. Screening was performed exclusively by FACS staining using the immunogen and the parental untransfected cells as positive and negative controls, respectively. More than 10,000 fusions were screened to generate about 20 clones, only a subset of which were further analyzed. mAb 2D7 is a previously characterized CCR5 antibody (14, 35) commercially available from PharMingen (San Diego, CA). CCR2-specific antibodies were obtained from R&D Systems. For FACS analysis, all antibodies were serially titrated against a high expressing 293-CCR5 stable cell line (293-R5–7) and used at a concentration of at least 2-fold above saturating concentrations.

FACS Analysis—Unless specified otherwise, 293T cells were transfected (via $CaPO_4$ precipitation) with either wild-type CCR5 or the appropriate mutant/chimeric construct and allowed to express for 18 h. Prior to primary antibody staining, transfected cells were lifted off with 2 mM EDTA, washed once with PBS, and incubated with FACS staining buffer (PBS, 2.5% calf serum, 0.5% BSA, and 0.02% sodium azide). Antibodies were added to a final concentration of 12.8 μg/ml, followed by secondary detection by affinity-purified phycoerythrin-conjugated horse anti-mouse antibody (1:100 dilution; Vector Laboratories). FACS analysis was performed on a Becton Dickinson FACScan flow cytometer using the CellQuest software (Becton Dickinson, San Jose, CA). The mean channel fluorescence (MCF) was used to compare the levels of coreceptor expression. Results were normalized for the MCF obtained for a particular antibody against wild-type CCR5 (normalized as 100%) after subtraction of the background MCF obtained against pcDNA3-transfected cells (normalized as 0%).

Cells and Proteins—293 and 293T cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (HyClone), 2 mM penicillin-streptomycin, and 2 mM glutamine. 293-CCR5 stables were made by transfecting (via $CaPO_4$ precipitation) 293 cells with pcDNA3-Zeo-CCR5 (a kind gift from Dennis Kolson) followed by 4 weeks of 0.3 mg/ml Zeocin (Invitrogen, Carlsbad, CA) selection. Foci that emerged after selection were plated together and the whole population subjected to FACS sorting for CCR5-positive cells using mAb 2D7. The highest expressing 5% of cells were sorted, expanded, and resorted by the same criteria to give high CCR5-expressing 293 cells. These cells are designated as 293-R5–7 cells. After 6 months of continual selection, these cells still gave a MCF value of >1000 on a background of <20 on the FL-2 channel when stained with phycoerythrin-conjugated 2D7 (PharMingen). Chemokines (RANTES, MIP-1β) were obtained from Preprotech. Recombinant vaccinia was used to produce soluble JRFL gp120. Briefly, recombinant vaccinia expressing soluble JRFL gp120 was used to infect 293T cells at 10 multiplicities of infection. Cells were exposed to virus for 4 h, washed twice with PBS, and replenished with serum-free Dulbecco's modified Eagle's medium. At 24 h after infection, the supernatant was harvested, clarified by centrifugation at 500 × *g*, filtered through a 0.45-μm filter, and inactivated with 0.2% Triton X-100. The recombinant protein was purified by lectin chromatography using *Galanthus nivalis* lectin-coupled agarose beads.

Chemokine/Env Blocking—^{125}I-Labeled MIP-1β or RANTES was purchased from NEN Life Science Products. Soluble JRFL gp120 produced as described above was iodinated using IODOGEN reagent according to the manufacturer's instructions (Pierce). Chemokine and Env binding was performed in Hepes binding buffer (HBB: 50 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$) with 0.5% and 5% BSA, respectively. A final concentration of 0.25 nM radiolabeled agonist (~70,000 cpm) was added to 0.5×10^5 293T cells transiently transfected with pcDNA3-CCR5. Concentrations of radiolabeled Env added can vary depending on the specific activity of the Env preparation, but ~100,000 cpm of Env was usually added per binding reaction. Binding was allowed to occur for 1 h at room temperature, and the radiolabeled agonist recovered on 25-mm GF/C glass microfiber filters (Whatman, Maidstone, United Kingdom) presoaked in 0.2% polyethyleneimine using a 10-well manual harvester following two washes with modified HBB (no BSA with 0.5 M NaCl). Filters were counted in a Wallac 1470

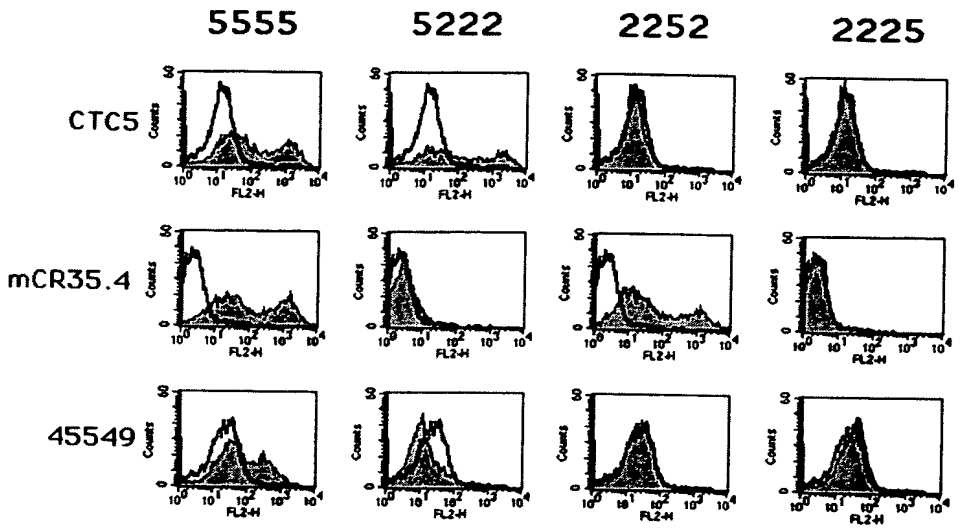

FIG. 1. **FACS analysis of different anti-CCR5 mAb against a panel of CCR5/CCR2 chimeras.** Chimeras are designated by their extracellular domains: *5555* is wild-type CCR5, *5222* designates a chimera with the N terminus of CCR5 and the first, second, and third extracellular loops of CCR2, etc. 293T cells were transfected with wild-type CCR5 or the appropriate chimeras, divided into equal aliquots of 500,000 cells, and stained with the various antibodies and detected with phycoerythrin-conjugated horse anti-mouse IgG. The background staining obtained with each antibody on pcDNA3-transfected 293T cells is overlaid on each histogram.

Wizard γ counter. Nonspecific binding was determined by the amount of counts recovered on the filter when no cells were used.

Virus Infection Studies—Luciferase reporter viruses were prepared as described previously (37, 36) by cotransfecting 293T cells with the indicated Env proteins and the NL4–3 luciferase virus backbone (pNL-luc-E^-R^-). Target cells used were GHOST cells stably expressing CCR5 (D. R. Littman, NIH AIDS Reference and Reagent Program). Infections were performed in 24 wells in the presence of 8 μg/ml Polybrene. At 4 days after infection, cells were lysed with 0.5% Triton X-100 in PBS and an appropriate aliquot analyzed for luciferase activity.

RESULTS

Epitope Specificities of Anti-CCR5 mAbs Fall into Three General Classes—Multiple domains of CCR5 participate in coreceptor and chemokine receptor function (31, 32, 34, 38–41). To more fully investigate CCR5 structure and function, we generated a panel of mAbs that could be used as immunological probes to identify regions of CCR5 important for Env and chemokine binding as well as for the induction of the conformational changes in Env that lead to membrane fusion. Since our previous attempts to generate anti-CCR5 antibodies by immunizing mice with peptides corresponding to CCR5 extracellular domains were disappointing, we immunized mice with cells stably expressing CCR5 in order to preserve the molecule's native conformation. CCR5-specific mAbs were identified by screening against CCR5-stable transfectants using the parental cell line as a negative control. Specificity was confirmed by testing the ability of each mAb to recognize cells expressing high levels of other chemokine receptors, including CCR2 to which CCR5 is most closely related (data not shown). A total of 18 mAbs to CCR5 were generated by this approach.

To identify domains of CCR5 to which each mAb was directed, we expressed a panel of CCR5/CCR2 chimeras in 293T cells and examined mAb reactivity to each by FACS. We first examined receptor chimeras in which a single extracellular domain of CCR5 was placed into a CCR2 background to determine if any of the mAbs bound to epitopes contained within any single extracellular domain of CCR5. We found three general classes of mAb epitope specificities: 1) N-terminal (NH_2) epitopes, 2) second extracellular loop (ECL2) epitopes, and 3) multidomain (MD) epitopes composed of more than one extracellular domain. For example, CTC5 was designated an NH_2-antibody because it recognized the chimera 5222, while mCR35.4 was designated an ECL2- antibody because it recognized 2252 (Fig. 1). Antibodies such as 45549 that did not recognize any single extracellular domain of CCR5 in the context of CCR2 (Fig. 1) were designated MD-antibodies (summarized in Table I). Interestingly, we did not find a single mAb that recognized ECL3 of CCR5 alone (2225), perhaps because the sequence of human CCR5 ECL3 is identical to that of the murine receptor. We confirmed that chimera 2225 was appropriately expressed on the cell surface, as determined by staining with a CCR2 mAb specific for the CCR2 N terminus (data not shown). However, we were unable to determine whether any of the mAbs recognized ECL1 alone because chimera 2522 was not well expressed (data not shown).

N-terminal mAbs Recognize Specific Residues in the Distal N Terminus of CCR5—mAbs that recognized 5222 with close to 100% efficiency relative to wild-type CCR5 were screened against N-terminal truncation mutants to gauge the extent of epitope coverage within the first 16 amino acids of CCR5. While some N-terminal mAbs recognized the Δ4 and Δ8 mutants (lacking the first 4 and 8 residues of CCR5, respectively), none recognized the Δ12 or Δ16 mutants (Table II). Expression of all N-terminal deletion mutants was confirmed by staining with ECL2 mAb 45531 (see Tables I and II). These results indicated that all N-terminal mAbs recognized residues within the first 13 amino acids of the CCR5 N-terminal domain, although this does not rule out contributions by residues in the proximal N terminus (after Asn-13). To more finely map residues important for antibody binding, the N-terminal mAbs were screened against a panel of CCR5 point mutants containing alanine or other non-synonymous substitutions from residues Asp-2 to Asn-13. Results from this analysis are summarized in Table II, and were in complete concordance with results obtained with the N-terminal truncations. For example, mAbs that did not recognize the Δ4 truncation mutant invariably failed to recognize the D3A, Y3A and/or Q4A mutants, while mAbs which recognized the Δ4 but not the Δ8 truncation mutant were affected by alanine substitutions such as S6A, S7A, or I9A.

ECL2 mAbs Recognize Several Immunodominant Residues in ECL2—ECL2 mAbs were screened against chimeras 22(25)2 and 22(52)2 to determine whether their epitopes lie within the first or second half of ECL2 (with the halves arbitrarily determined by Cys-178; Fig. 7*C*). Of the five ECL2 antibodies examined, only one, 2D7, recognized the first half of ECL2 (ECL2-A) while the rest recognized the second half of ECL2 (ECL2-B) (Table I). The ECL2 of human and mouse CCR5 differ by only six amino acids, five of which are located in ECL2-B (*red-circled* residues in Fig. 7*C*). When tested against a series of ECL2-B alanine scan mutants, we found that mAbs 45529, 45531, mCR35.4, and mCR40.3 were all dependent upon residues Tyr-184 to Phe-189 (Fig. 2*A*) where the majority of mouse-human differences are located. mAbs 45529, 45531, mCR35.4, and

mCR40.3 were made from two different strains of mice (BALB/c and AKR) using two different immunogens (NSO-CCR5 stables and CHO-CCR5 stables), yet mapped to identical epitopes. This suggests that residues Tyr-184 to Phe-189 represent an immunologically accessible and antigenically dominant face of CCR5 (*yellow* residues in Fig. 7*C*). On the other hand, 2D7 recognition of CCR5 was prevented by the single point mutants K171A and E172A located in ECL2-A (Fig. 2*B*). Curiously, binding of all MD mAbs was also prevented by these two point mutants, suggesting that residues Lys-171/Glu-172 represent another immunologically accessible area in ECL2 (see Table III) that, judging by its importance for MD mAbs, is in close association with other ECLs.

Characterization of MD mAbs—MD mAbs were screened against a panel of CCR5/CCR2 chimeras where single extracellular domains of CCR5 were sequentially replaced by the homologous regions from CCR2. As shown in Fig. 3, all MD mAbs recognized chimera 2555 indicating that none of them have an absolute requirement for the intact N terminus of CCR5. On the other hand, the epitopes recognized by the MD antibodies involved ECL2 as none recognized chimera 5525 even though it was well expressed on the cell surface. Some of these mAbs were also dependent upon ECL1 (*e.g.* 45501 and 45533) as recognition of the 5255 chimera by these mAbs was less than 20% of the wild-type CCR5 value. Contributions of ECL3 to any of the MD mAb epitopes were difficult to access as the 5552 chimera was not well expressed (Fig. 3). When tested against a panel of ECL2 point mutants, we found that recognition of CCR5 by all MD mAbs was eliminated by the K171A and E172A mutations (Table III). None of the other alanine scan mutants in ECL2 affected CCR5 recognition by these mAbs.

TABLE I
Summary of epitope specificities of selected anti-CCR5 mAb

Antibody	Epitope specificity			
	NH_2	ECL2		MD
CTC2	+	−		−
CTC5	+	−		−
CTC8	+	−		−
CTC9	+	−		−
CTC12	+	−		−
CTT14	+	−		−
45502	+	−		−
45519	+	−		−
		ECL2-A	ECL-2B	
45529	−	−	+	−
45531	−	−	+	−
mCR35.4	−	−	+	−
mCR40.3	−	−	+	−
2D7	−	+	−	−
45501	+/−[a]	+[b]		+
45517	−	−[c]		+
45523	+/−[a]	+[b]		+
45533	−	−[c]		+
45549	−	−[c]		+

[a] May have minimal recognition of CCR5 N terminus although N terminus is neither necessary (2555 is recognized) or sufficient for its epitope.

[b] Considered a MD antibody even though it may posses moderate recognition of ECL2 alone (2252) because removal of ECL1 or mutations of particular residues in ECL1 impairs epitope recognition.

[c] Considered a MD antibody because removal of ECL2 abrogates epitope recognition, but ECL2 alone (2252) is not sufficient for full epitope recognition.

Recognition of Linear Epitopes on CCR5 Is Rare—We also sought to determine if any of the anti-CCR5 mAbs were able to detect CCR5 by Western blot analysis as an indication of a conformation independent epitope. We tested all N-terminal mAbs and select ECL2 and MD mAbs and found only two (N-terminal mAbs CTC5 and CTC8) capable of Western blotting CCR5 efficiently (Fig. 4 and data not shown). The specificity of these mAbs for denatured CCR5 was shown by the fact that single point mutants that prevented recognition by CTC5 and CTC8 in FACS assays (D2A and D11A respectively, see Table II) also prevented recognition by Western blot (Fig. 4). It is also notable that recognition of CCR5 by CTC5 was eliminated by a 9-amino acid HA tag engineered to follow the initiator methionine in CCR5. While MD mAbs would not be expected to recognize denatured CCR5, we found it surprising that the majority of N-terminal mAbs were also unable to recognize CCR5 by Western blot, suggesting that even the N terminus of CCR5 is structurally complex, perhaps being held in position by specific interactions with other CCR5 domains. Alternatively, our definition of N-terminal mAbs may include some that recognize ECL residues conserved between CCR2 and CCR5. However, this is unlikely given that the entire panel of N-terminal mAbs also recognized receptor chimeras in which the CCR5 N-terminal domain was placed in CCR1, CXCR2, or CXCR4 backgrounds (data not shown).

Lack of Agonist Activity—Although antibodies with agonist activity are rare, it has been reported at least once for an antibody directed against CCR2 (42, 43). Since we had mAbs directed against multiple regions in CCR5, some of which appeared to overlap the chemokine binding sites in CCR5 (see below), we determined if any of our mAbs possessed agonist activity. Using a highly sensitive aqueorin-based system for detecting Ca^{2+} flux (44), we tested the CCR5 mAbs singly or in

TABLE II
Epitope mapping of N-terminal antibodies

Antibodies which reacted only against the 5222 chimera in Table 1 were screened against N-terminal deletion mutants. Consecutive four-amino acid deletion were made after the initiator methionine (Δ4) up to residue 17 (Δ16), and these deletion mutants were used in FACS analysis with the listed N-terminal antibodies. All the N-terminal deletion mutants were expressed at 50–150% of wild-type CCR5 levels as judged by ECL2 antibodies (2D7 and 45531, data for 45531 are shown). Results are presented normalized against the MCF value for the wild-type CCR5 obtained with each antibody (set at 100% after subtraction of the background (MCF value obtained for each antibody against pcDNA3-transfected cells); background MCF values are typically less than 5–10% of CCR5 MCF values and never greater than 20% of CCR5 MCF for any of the antibodies tested). Shaded boxes represent recognition of point mutants at less than 50% of wild-type CCR5 levels. 45531 is an ECL2 antibody used to control for expression levels among the different N-terminal truncation and point mutants. −, <10% CCR5 MCF; −/+, ≥10% CCR5 MCF; +, ≥50% CCR5 MCF; ++, ≥100% CCR5 MCF; +++, ≥150% CCR5 MCF; ++++, ≥200% CCR5 MCF.

	N-terminal Truncations				N-terminal Alanine Scan Mutants											
Antibody	Δ4	Δ8	Δ12	Δ16	D2A	Y3A	Q4A	V5A	S6A	S7A	P8A	I9A	Y10A	D11A	H12A	N13D	CCR5
CTC5	-	-	-	-		+	++	++	++++	++++	+++	+++	++	++	++	++	++
CTC8	-	-	-	-			+++	+++	++++	++++	++++	++++	++	++	++	++++	++
502	-	-	-	-			+	++	++++	+++	++++	+++	++	++	++	++	++
519	-	-	-	-				+++	++++	++++	+++	+++	++	++	++	++++	++
CTC12	++	-	-	-	++	++	+++	++	++++		+++				++	++	++
CTC3	+++	-	-	-	++	+++	+++	++			-	+			+	++	++
CTC4	++	+	-	-	++	++++	++	++	+++	+++	++	+++					++
45531(ECL2)	++	++	++	+++	++	++++	++	++	++++	+++	++++	+++	++	++	++	+++	++

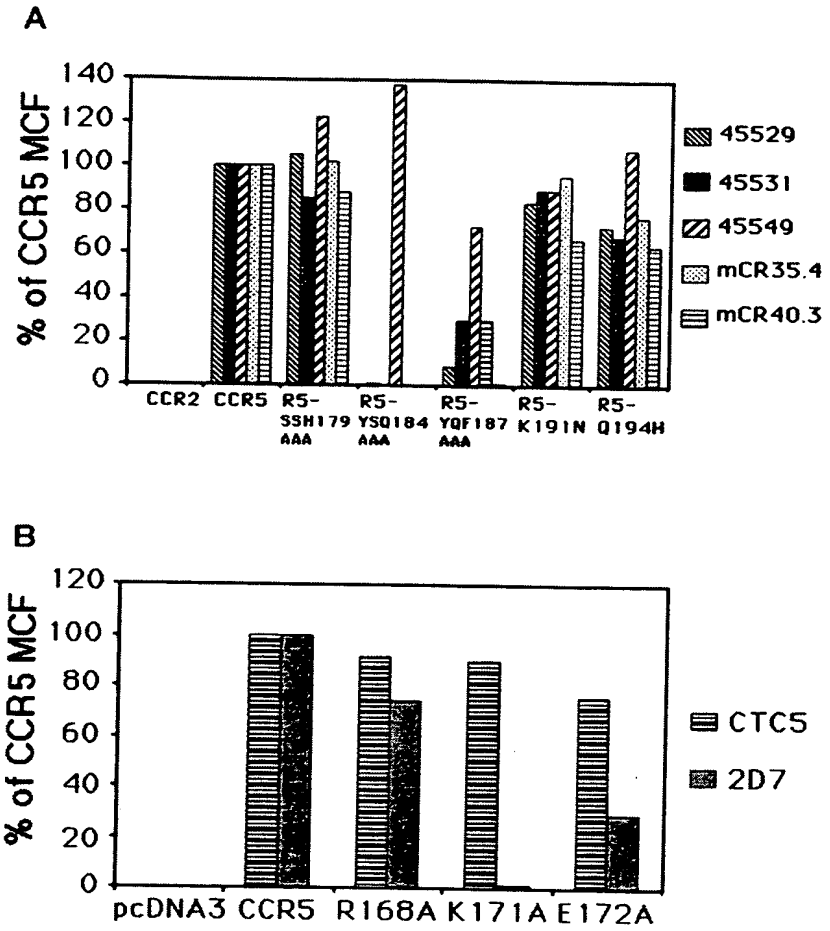

FIG. 2. **Fine epitope mapping of ECL2 antibodies.** *A*, ECL2 antibodies that reacted against the second half of ECL2 (termed ECL-2B, see Table I) were screened against alanine scan mutants spanning residues Ser-179 to Phe-189 as well as the K191N and Q194H point mutants. Results are normalized to the MCF value for wild-type CCR5 obtained with each antibody (set at 100%). 45549 is a MD, conformationally sensitive antibody whose epitope involved the first half of ECL2 (Table III and data not shown) and is used here to control for coreceptor expression. All FACS analyses were performed two to three independent times, and values did not vary from the data shown by more than 10%. *B*, 2D7, directed against ECL2-A, was screened against alanine scan mutants of selected residues in that region. Results are normalized as in *A*.

TABLE III
Epitope mapping of MD mAbs

All point mutants were expressed at greater than 50% of wild-type CCR5 levels as judged by FACs analysis with either N-terminal antibodies (CTC5 and CTC8) or ECL2 antibodies (2D7 and 45529). ND, not determined. −, <10% CCR5 MCF; +/−, ≥10% CCR5 MCF; +, ≥50% CCR5 MCF; ++, ≥100% CCR5 MCF.

Antibody	ECL2-A			ECL2-B			ECL1		
	E168A	K171A	E172A	K191A	Q194N	K197A	A92N	Q93A	D95A
45501	++	−	−	++	++	++	++	++	++
45517	++	−	−	++	++	++	ND	ND	ND
45523	++	−	−	++	++	++	++	++	+/−
45533	++	−	−	++	++	++	+	+	+
45549	++	−	−	++	++	++	++	+	+

combination for their ability to induce a Ca^{2+} flux in CHO-CCR5 stable cell lines. None of the antibodies or combinations of antibodies tested induced a Ca^{2+} flux (data not shown).

Blockade of Chemokine and Coreceptor Function—Available evidence indicates that chemokines and HIV-1 Env bind to CCR5 using overlapping but distinct domains (34, 35). To extend these findings, we tested the ability of a panel of CCR5 mAbs to inhibit binding of ^{125}I-RANTES and MIP-1β (Fig. 5*A*) or ^{125}I-labeled soluble HIV-1 JR-FL gp120 (Fig. 5*B*) to CCR5. mAb 2D7 was efficient at blocking both chemokine and gp120 binding to CCR5, suggesting that ECL2-A is crucial for both events. However, N-terminal mAbs (CTC5 and CTC8) blocked gp120 binding much more efficiently than chemokine binding, while mAbs directed against ECL2-B (45529 and 45531) blocked chemokine binding more efficiently than gp120 binding. These results suggest that chemokines are more dependent on ECL2 (specifically residues Tyr-184 to Phe-189), and soluble monomeric gp120 more dependent on the N terminus, for binding to CCR5. MD mAbs were variable in the extent they blocked chemokine or Env binding. We believe that this differential blockade is due more to epitope specificities of the antibodies rather than their affinities for CCR5, as will be shown below.

Differential Blockade of Env Binding and Virus Infection—We anticipated that mAbs which blocked gp120 binding would also inhibit infection of CCR5-positive cells by R5 virus strains. Using luciferase reporter viruses in infection studies on GHOST-CCR5 cells, we found that mAb 2D7 blocked R5 virus infection (ADA, JRFL, and BaL, data not shown for ADA) to the same extent as has been previously reported (Ref. 35 and Fig. 5*C*). However, none of the other mAbs consistently blocked virus infection by more than 40–50%, even at concentrations as high as 20 μg/ml (Fig. 5*C*). Most strikingly, N-terminal mAbs that inhibited gp120 binding by up to 80% were not as efficient as other ECL2 or MD antibodies in blocking virus infection (compare CTC5 in Fig. 5, *B* and *C*). ECL2 antibodies in general, and 2D7 in particular, inhibited viral infection by 40–70% whereas all N-terminal antibodies tested (CTC5, 8, and 12) inhibited viral infection only by about 10–40% (Fig. 5*C* and data not shown). Similar results were obtained when infection was done on PM1 cells (data not shown). Thus, the ability of a mAb to inhibit binding of soluble, monomeric gp120 to CCR5 did not accurately predict virus neutralization activity. A model to account for these results is that while the N terminus of CCR5 provides the initial binding site for gp120, the extracellular loops are more important for inducing the conforma-

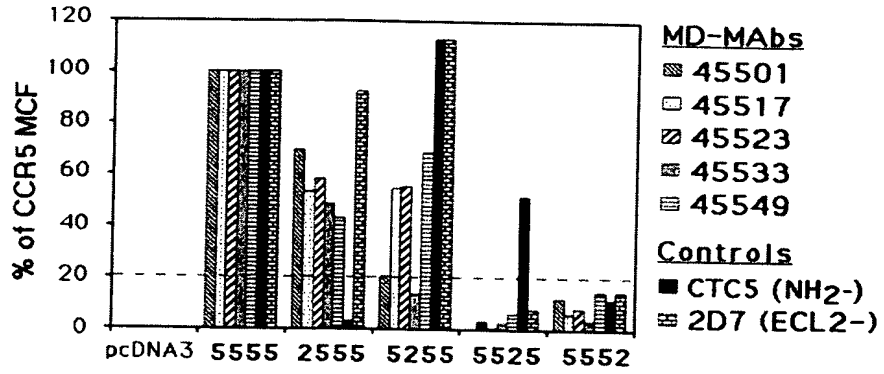

FIG. 3. **Epitope mapping of MD antibodies.** mAbs that did not react against any single CCR5 extracellular domain in the context of CCR2 (Table I) were screened against CCR5/CCR2 chimeras where a single extracellular domain of CCR5 was sequentially replaced with the homologous CCR2 region. CTC5, an N-terminal antibody, and 2D7, an ECL2-A antibody, were used to control for receptor expression. All chimeras were expressed at greater than 50% of wild-type CCR5 levels except for the 5552 chimera. The *dashed line* represents the 20% mark.

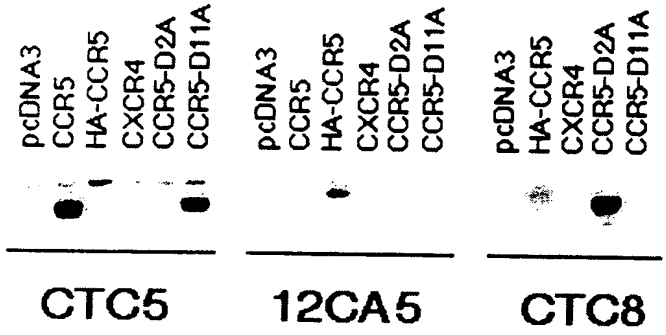

FIG. 4. **Western blot of CCR5 using N-terminal mAbs.** CTC5 and CTC8 were the only CCR5-specific mAbs in our panel which efficiently recognized CCR5 by Western blot. 293T cells were transfected with the various constructs indicated (all in pcDNA3), and clarified cell lysates equivalent to approximately 2.5×10^5 cells were loaded per lane. These cell lysates were run on an 8% SDS-polyacrylamide gel with 4 M urea, transferred onto polyvinylidene difluoride, membranes and blotted with ≤ 0.5 μg/ml of the indicated antibody. 12CA5 is a mAb specific for the HA tag. HA-CCR5 is CCR5 with a 9-amino acid HA tag on the N terminus.

tional changes in virion-associated Env that lead to membrane fusion.

CCR5 Exists in Multiple Conformational States—While the ability of 2D7 to inhibit virus infection more efficiently than other CCR5 mAbs could be due to its unique pattern of CCR5 recognition, the results might also be explained by differences in antibody affinity or on-rate, since cells were preincubated with mAb for 1 h prior to the addition of CCR5 ligand. Therefore, we measured the approximate affinity of each mAb for CCR5 by serially diluting each antibody from 12.8 μg/ml to 0.1 μg/ml prior to staining 293-R5-7 stable cells. To account for minor variations in day-to-day staining and flow cytometer calibration, the EC_{50} values for each mAb (defined as the concentration of antibody which gave half-maximal MCF value) were normalized to that observed with mAb 2D7 in each experiment (nEC_{50}, see legend to Fig. 6). Thus, an antibody with an nEC_{50} of 100% would have a relative affinity identical to that of 2D7, while an antibody with an nEC_{50} of 1000% would have a 10-fold lower affinity. As can be seen, some N-terminal (CTC5 and CTC8) and MD antibodies (45523, 45533) had equal or greater affinities than 2D7 (<100% of 2D7 nEC_{50}) while other ECL2 antibodies (*e.g.* 45529 and mCR40.3) had greater than 10-fold lower affinities (>1000% of 2D7 nEC_{50}). In addition, 2D7 binding to CCR5 reached equilibrium by 15 min, similar to most other mAbs (Fig. 6). However, we noted that 2D7 consistently gave maximal MCF values almost 2–6-fold higher than that of any other mAb. This greater reactivity with cell surface CCR5 could not simply be explained by differences in on-rate, antibody affinity, or isotype, and instead suggests that 2D7 recognizes a greater proportion of CCR5 molecules on the cell surface than other CCR5 mAbs, including some mAbs with higher relative affinities. Thus, we conclude that 2D7 reacts with a unique conformation-dependent determinant that is comprised at least in part by the first half of ECL2 (Fig. 7*C*), and that this determinant is present on a greater proportion of CCR5 molecules than are other epitopes. It is not clear if the multiple conformational states we have observed are due to interactions between CCR5 and other cell surface proteins that modulate epitope exposure.

DISCUSSION

CCR5 is of paramount importance for HIV infection, given the resistance of individuals who lack this chemokine receptor to virus transmission (8–10, 45). Dissecting the structural domains of CCR5 required for chemokine binding, Env binding, and for induction of conformational changes in Env that lead to membrane fusion will facilitate the rational development of CCR5 antagonists to prevent viral entry. In this study, we generated a large panel of mAbs to investigate the structural organization of CCR5's extracellular domains. Fine epitope mapping of 18 mAbs to CCR5 revealed only a few sites that were antigenically dominant and immunologically accessible. The distal N terminus (amino acids 2–13) and ECL2 contributed to the majority of the epitopes recognized by the panel of mAbs, although the homology between human and murine CCR5 may limit what epitopes of CCR5 are immunogenic (39, 40, 46). For example, the majority of ECL2 mAbs mapped to the second half of ECL2, specifically to the region where the majority of differences between human and mouse CCR5 occur (amino acids 184–189, see Fig. 7*C*). Another dominant immunologic feature in ECL2 involved residues Lys-171 and Glu-172. Recognition of CCR5 by all MD antibodies, as well as 2D7 (directed against ECL2-A), was severely compromised by point mutations in either of these two residues. The importance of these residues for all MD antibodies suggests that K171 and E172 form part of a bridge between the ECL2 and another ECL. ECL1, to which ECL2 is disulfide-bonded, is an obvious candidate.

The N-terminal domain of CCR5 also made major contributions to a number of antigenic epitopes. Interestingly, this domain also appears to be structurally complex, as indicated by the rarity of N-terminal mAbs which recognized denatured CCR5 as judged by Western blot analysis. This may mean that interactions with other extracellular domains of CCR5 are required to maintain the proper conformation of the N terminus. In fact, a highly conserved Cys residue at position 20 in the CCR5 N terminus is thought to form a disulfide bond with a Cys residue in ECL3. That the CCR5 N-terminal domain exhibits conformational complexity is also supported by observations that mutations in ECL1 can affect epitope recognition by some N-terminal antibodies (47) and our findings that some point mutations involving charged residues in the extracellular

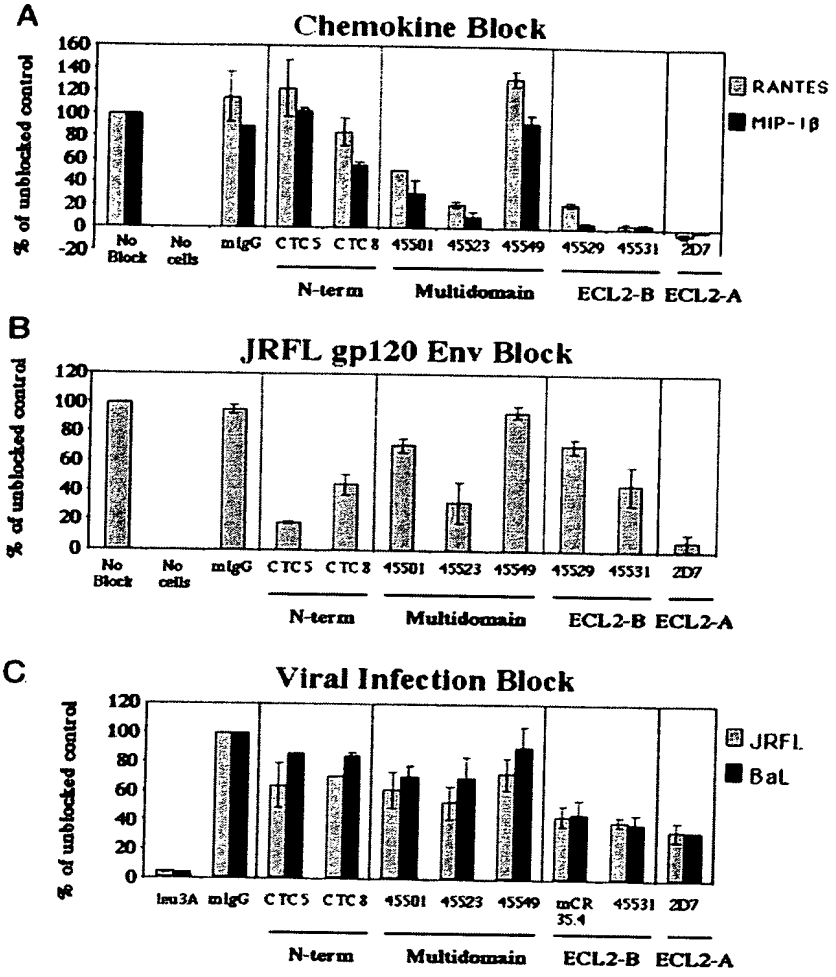

FIG. 5. **Differential blockade of chemokine, soluble JRFL gp120 Env binding, and viral infection.** Selected N-terminal, MD, or ECL2 antibodies were tested for the ability to block either ^{125}I-labeled RANTES/MIP-1β (*A*), ^{125}I-labeled soluble JRFL gp120 binding (*B*) or R5-Env pseudotyped luciferase reporter virus infection of Ghost-CCR5 cells (*C*). 10 μg/ml amounts of the indicated mAbs were preincubated for 1 h with either 2×10^5 293T cells previously transfected with CCR5 plasmid (*A* and *B*) or 2.5×10^5 Ghost-CCR5 cells (*C*). Soluble gp120 binding was performed in the presence of 100 nM soluble CD4. Results are normalized to the number of counts obtained with the unblocked control (*A* and *B*) or the relative light units (*RLU*) obtained by infection with the luciferase reporter viruses in the presence of normal mouse IgG (*C*). Data are shown as averages ± S.E. from at least three independent experiments.

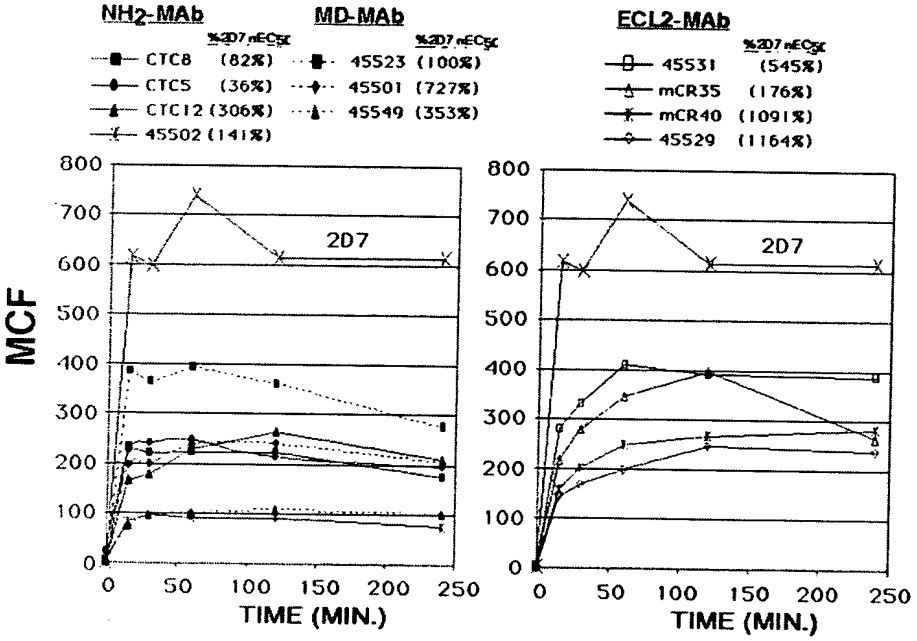

FIG. 6. **Equilibrium binding of anti-CCR5 mAbs.** Selected mAbs were used at saturating conditions (12.8 μg/ml) to stain 293-CCR5 stable cells. The primary mAbs were allowed to stain 293-CCR5 stable cells for the indicated times before washing and staining with the secondary detection antibody. Addition of the primary antibody was staggered so that the secondary detection antibody was added at the same time. *Left panel* shows 2D7 with N-terminal and MD mAbs; *right panel* shows 2D7 with other ECL2 mAbs. Each time point with each mAb was repeated twice with similar results. Indicated in *parentheses* are the relative affinities for each antibody presented as normalized EC_{50} values (nEC_{50}). The EC_{50} value of each antibody was the concentration of antibody that gave half-maximal MCF value based upon FACS analysis using serial dilutions of the mAb onto 293-CCR5 stable cells. To account for minor variations in day to day staining and flow cytometer calibration, 2D7 was serially titrated in every experiment and all EC_{50} values were normalized against the EC_{50} value of 2D7, which was set at 100%.

loops sometimes result in greater reactivity by N-terminal mAbs (data not shown). Although we cannot rule out the possibility that some NH_2 mAbs directly interact with residues in other CCR5 domains, the ability of our entire panel of N-terminal mAbs to recognize additional receptor chimeras in which the CCR5 N-terminal domain is placed in CCR1, CXCR2, or CXCR4 backgrounds makes this possibility less likely (data not shown).

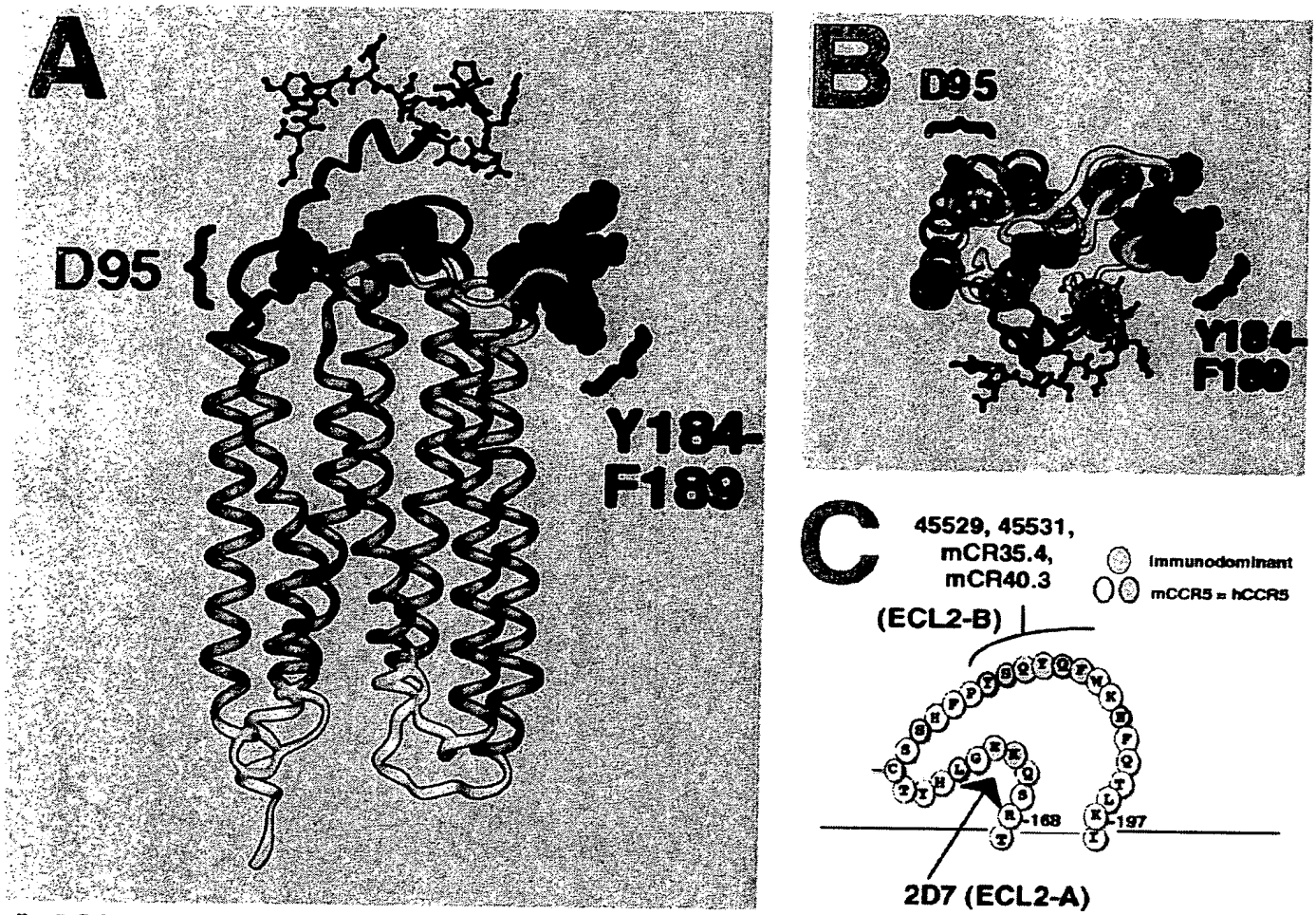

FIG. 7. **Schematic model of CCR5.** A hypothetical model of CCR5 was generated by initial placement of the helices based on the cryo-electron micrographs of rhodopsin (56, 57). The structure was then modified to be in accord with the available physically determined structural data, and with general physiochemical criteria (involving patterns of residue polarity and conservation) that we use for modeling membrane proteins (see Ref. 58). Transmembrane helixes are indicated in *cyan*. The extracellular loops of CCR5 are color coded: N terminus, *blue*; ECL1, *green*; ECL2, *yellow*; ECL3, *magenta*. The intracellular loops are indicated in *white*. The first 13 amino acids in the N terminus are indicated by the *ball-and-stick* representations. Immunodominant residues (171–172, 184–189) in ECL2 are indicated by the *red* space-filling residues. Asp-95 is indicated by the *green* space-filling residue. *A*, a side view of CCR5 along the plane of the plasma membrane. Note that the side chains of residues 184–189 are pointed away from the main body of the molecule, a plausible orientation that makes it accessible to antibody binding. *B*, a top-down view of CCR5, observing from the extracellular space toward the plasma membrane. Note that residues 184–189 still look highly accessible, as is residue Asp-95. The N terminus is placed in a manner that makes Asp-95, Lys-171, and Glu-172 accessible as a potential binding site for an antibody (*e.g.* mAb 45523). *C*, diagrammatic representation of ECL2 of human CCR5. *Red-circled* residues represent differences between human and mouse CCR5, *green-circled* residue represents the conserved cysteine in ECL2, and *yellow* shaded residues represent the antigenically accessible residues recognized by ECL2 and other MD antibodies.

Identification of antigenic epitopes can be used to provide constraints on theoretical models of CCR5. Residues involved in antibody binding are likely to be surface-accessible, while residues in different extracellular domains that comprise a single antigenic determinant are likely to be in close spatial proximity as a consequence of the way in which CCR5 folds. For example, Fig. 7 (*A* and *B*) shows the placement of residues Tyr-184 to Phe-189 (*red* space-filling residues) on a hypothetical CCR5 model that takes into consideration data presented in this study. It is notable that the β-sheet proposed for ECL2 in this model places Tyr-184 to Phe-189 on an easily accessible face of CCR5, which is consistent with these residues contributing to the epitopes recognized by ECL2-B mAbs. In addition, residues Lys-171 and Glu-172 (unlabeled *red* space-filling residues) must also be accessible since all MD mAbs presumably require contact with these two amino acids (Table III). Finally, mAb 45523 is dependent upon residues Asp-95, Lys-171, and Glu-172 (Table III), and these residues are positioned in close proximity. Studies such as this will lead to additional refinements of structural models that can, in turn, be used to generate testable hypotheses regarding CCR5 structure and function.

We also used the fine epitope maps of these mAbs to study how chemokines and HIV-1 Env bind to CCR5. The differential blockade of chemokine *versus* Env binding to CCR5 by some mAbs cannot be explained simply by differences in affinity since the relative EC_{50} values for each antibody did not correlate with the ability to block ligand binding. For example, the ECL2B mAb 45529 blocked chemokine binding more efficiently than the NH_2 mAb CTC5 despite having a markedly lower nEC_{50} value. Rather, the ability of a given mAb to block Env or chemokine binding correlated with the domain of CCR5 to which the mAb bound. Thus, NH_2 mAbs that did not block chemokine binding (CTC5 and CTC8) blocked gp120 binding quite efficiently, while ECL2-B mAbs blocked chemokine binding but were relatively inefficient at blocking gp120 binding (Fig. 6). This is consistent with work reported by Wu *et al.* (35) using a different set of anti-CCR5 mAbs. The ability of ECL2-B

mAbs to block RANTES and MIP-1β binding is also consistent with reports indicating that ECL2 of CCR5 is important for chemokine binding (48) and that alanine mutants of residues His-181 and Tyr-184 are impaired in their ability to bind RANTES and MIP-1β (34). Interestingly, Wu *et al.* (35) also reported that an N-terminal mAb (3A9) efficiently blocks gp120 binding, which is consistent with our findings. Together, these results argue that Env and the β-chemokines interact with overlapping but distinct sites on CCR5.

We hypothesized that there would be a correlation between the ability of a mAb to block gp120 binding and its ability to block HIV infection. However, this proved not to be the case. In general, mAbs directed against ECL2 neutralized HIV more effectively than N-terminal mAbs despite the fact that they typically inhibited binding of monomeric gp120 to CCR5 very poorly. We speculate that, in the context of virus infection, membrane-bound CD4 and, possibly, other cell-associated adhesion molecules (49–53) provide the initial attachment of the virus to the cell surface, thereby making direct interactions between Env and the CCR5 N-terminal domain less important for coreceptor function. Indeed, although the N-terminal domain of CCR5 is clearly important for gp120 binding (Ref. 34 and data not shown), and antibodies to this domain inhibit this interaction (Fig. 5), many viruses can tolerate mutations or even significant truncations of this domain (31, 47). By contrast, regions in the extracellular loops of CCR5, particularly ECL2, are critically important for coreceptor function, suggesting that these domains are important for triggering the fusogenic conformational changes in Env. Previous studies indicate that there are indispensable residues in ECL2 (Pro-183, Tyr-184, Ser-185) that are required for coreceptor function (4, 46, 54). Thus, our viral inhibition data are consistent with structure-function data generated from mutagenesis experiments.

G-protein-coupled receptors can exist in multiple, interconvertible receptor affinity states. This is particularly well characterized for β-adrenergic receptors, where agonist competition profiles can vary depending on the degree of G-protein coupling and the concentration of guanine nucleotides present in the system (reviewed in Ref. 55). Our kinetic analyses of mAb binding activity using saturating antibody concentrations revealed equilibrium binding states with varying levels of plateau MCF values (Fig. 6). This differential reactivity with cell surface CCR5 under saturating antibody concentrations could not be ascribed to either affinity or kinetic differences. Rather, we believe that the variable MCF values obtained with the panel of mAbs under saturating, equilibrium binding conditions are due to different CCR5 conformational states. Alternatively, CCR5 may associate with other cell surface molecules that affect CCR5 conformation or which mask particular epitopes. However, it is important to note that we did not observe any differences in antibody reactivity to CCR5 when CD4 was coexpressed (data not shown). These findings may help explain the unique ability of mAb 2D7 to block both chemokine and Env binding more efficiently than all other mAbs tested, even those with equal or greater affinities for CCR5 (*e.g.* 45523, CTC5, CTC8). In addition, 2D7 was the mAb that neutralized HIV more effectively than any of the other mAbs examined. Notably, 2D7 bound to a much greater fraction of cell surface CCR5 molecules than other mAbs, suggesting that its epitope is present and accessible on a large fraction of CCR5 molecules. The epitope recognized by 2D7 was also unique, involving the first half of ECL2 (ECL2A). Thus, the ability of 2D7 to inhibit chemokine binding, Env binding, and viral infection may reflect both its unique epitope (ECL-2A: Lys-171/Glu-172), its relatively high affinity for CCR5, and the fact that it reacts with a larger proportion of cell surface CCR5 molecules than other antibodies. Consequently, the ECL2-A to which 2D7 is directed may be a particularly important region of CCR5 to target during the development of receptor antagonists.

In summary, we have mapped the epitopes recognized by a large panel of anti-CCR5 mAbs to study the structure and function of CCR5. We believe this approach complements mutagenic techniques for studying CCR5 structure and function and will provide constraints on the theoretical modeling of CCR5. However, the lack of antibodies directed against more conserved regions of CCR5 is a current limitation of this approach. Therefore, production of second generation antibodies against CCR5 has been initiated using CCR5 knock-out mice. We hope that anti-CCR5 antibodies generated in these mice will exhibit greater epitope diversity than those currently available. Of particular interest is the finding that CCR5 can exist in multiple conformational states. The nature of these conformational states, whether they are interconvertible, whether each can support chemokine or coreceptor activity, and the impact of cell type on antibody reactivity remain to be determined. Does CCR5 exist in distinct conformations, or might some fraction of cell surface molecules associate with other proteins, perhaps obscuring some antigenic determinants? The results hint at some of the complexities of CCR5 structure and presentation that may not yet be fully appreciated when evaluating chemokine receptor expression on the surface of primary cells.

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